MOLECULAR CROWDING EFFECTS ON STABILITY AND KINETICS OF TRINUCLEOTIDE REPEAT HAIRPINS

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ABSTRACT

Numerous genetic disorders arise from the propensity of certain repetitive DNA sequences to form intrastrand, non-helical structures, such as hairpins, due to extensive self-complementarity. Within the cellular environment, the formation of these non-helical structures occurs within highly crowded conditions. Using single-molecule FRET microscopy, the effect of molecular crowding on the stability and dynamics of individual DNA hairpins containing the trinucleotide repeat sequences $(CAG)_5$ and $(CTG)_5$ were explored. These repeat sequences have central mismatches within each repeat unit that reduce the stability of intrastrand contacts between repeat units in the stem, leading to highly dynamic behavior for their hairpins. The structural dynamics for $(CAG)_5$ and $(CTG)_5$ trinucleotide repeat hairpins were quantified in the presence of molecular crowding agents (polyethylene glycol; PEG) with different sizes. Analysis of the transitions rates between the unstructured and closer conformations shows that an increase in molecular crowding promotes the formation of the hairpins. Strikingly, the crowding agents induce a greater acceleration of hairpin melting and overall destabilization, which contrasts with observations from controls with fully paired DNA hairpins. Under these conditions, the high mismatch content of these repeat hairpins becomes more destabilizing in the presence of PEG. The findings indicate that molecular crowding may confer some protective benefit to genomic DNA by disrupting these deleterious structures at smaller repeat sizes.

OVERVIEW OF TRINUCLEOTIDE REPEAT DISORDERS

DNA stores our genetic information; however, external factors and normal cellular processing of DNA can introduce errors that compromise its information. Fortunately, the cell has numerous correction mechanisms to protect its genomic integrity. Some errors cannot be readily repaired or produce responses that worsen the error. An important example of this occurs with the expansion of trinucleotide repeats (TNR) and other repetitive DNA elements. TNRs refer to the repetition of a specific sequence of bases (such as CGG or CAG) at certain genetic loci. TNRs have been linked to several diseases, including Fragile X syndrome, Huntington's disease, and spinocerebellar ataxias. Early structural studies of DNA containing trinucleotide repeats identified the presence of non-helical (alternative) structures within the DNA (2). The primary structure is the hairpin, which results from one DNA strand folding back on itself due to the self-complementarity of these repeat regions. These hairpins do not have full Watson-Crick base pairing but instead have a mismatch located in the middle position of each repeat with CTG repeats more stable than CAG repeats due to the higher stability of T·T mismatch compared to the A·A mismatch. Pathogenesis likely initiates due to interference from hairpins and other alternative structures with DNA processing and its repair due to DNA damage.

smFRET APPROACH TO STUDY TNR HAIRPINS

The experiments examined the conformational changes of trinucleotide repeat-containing DNA hairpins that occur through formation and melting of intramolecular base pairs. The hairpins can exist in two forms: an open (unpaired) state and a closed (base paired) state (Figure 1). Single-molecule fluorescence resonance energy transfer (smFRET) provides a powerful approach to monitor directly which conformation the hairpins are populating. For this method, the hairpins were constructed from two synthetic strands of DNA, each labeled with a different fluorescent molecule - Cy3 and Cy5. These fluorescent molecules comprise a FRET donor-acceptor pair. For this approach, the Cy3 dye is directly excited, and Cy3 transfers energy to Cy5 in a distance-dependent manner. The relative amounts of emitted light by Cy3 and Cy5 are correlated to the distance between the dyes, which can then serve as a signal for whether the hairpin is in the open or closed state. An individual hairpin emits primarily light corresponding to Cy5 in the closed state and to Cy3 in the open state. This setup provides a discrete signal for each conformation. Using widefield total internal reflection microscopy (TIRFM), discrete dye signals data are acquired from hundreds of individual molecules simultaneously.

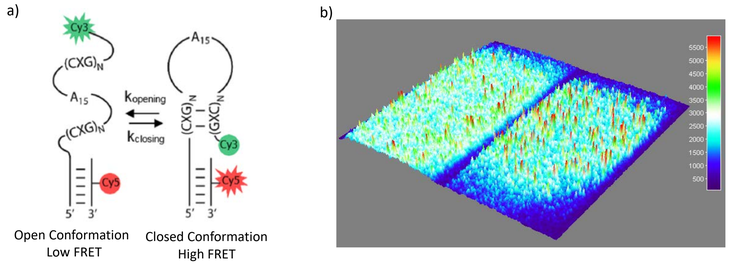

Figure 1. Illustration of the two detectable FRET conformations for DNA hairpins. a) In the closed conformation, the fluorescent dyes are approximately 3 nm apart. In the open conformation, the distance between the dyes increases to 9 nm. b) b) Representative image of a single widefield of view for $(CTG)_5$ at 25 mM NaCl showing Cy3 fluorescence (left side) and Cy5 fluorescence (right side).

CONSTRUCT DESIGN FOR DNA TNR HAIRPINS AND smFRET BEHAVIOR

The DNA hairpins were designed with a specified number of trinucleotides repeats, $(CAG)_5$ and $(CTG)_5$, that have a mismatch in the central position. smFRET time traces showed resolvable fluctuations between the open and closed forms.

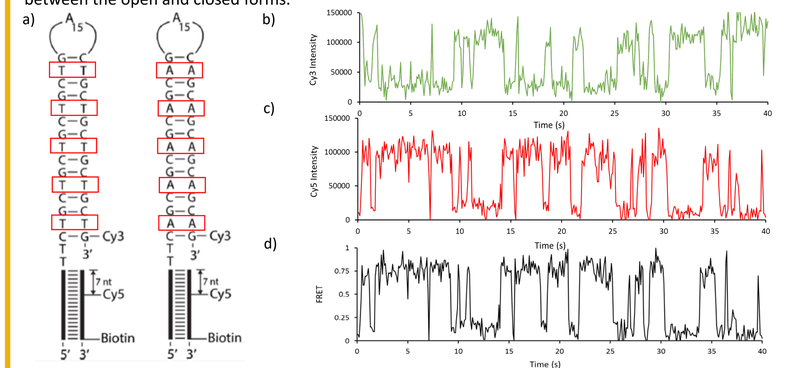

Figure 2. a) Sequences for DNA hairpin constructs $(CTG)_5$ and $(CAG)_5$. b) – d) Representative time trace for a single $(CTG)_5$ observed for 40 s in 25 mM NaCl. For each molecule, the (b) Cy3 and (c) Cy5 intensities were collected. The (d) FRET trace is calculated as $FRET(t) = \frac{\beta I_{Cy5}(t)}{I_{Cy3}(t)+I_{Cy5}(t)}$.

SALT-DEPENDENCE OF TNR DYNAMICS AND CROWDING EFFECTS

Monovalent salt shifts the equilibrium toward the hairpin conformation by slowing the opening transition. In contrast, molecular crowding by PEG accelerates both the opening and closing transitions.

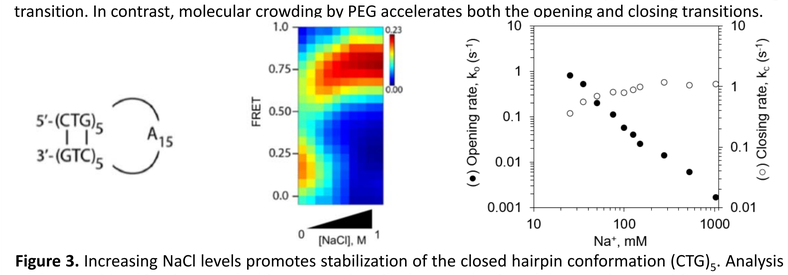

Figure 3. Increasing NaCl levels promotes stabilization of the closed hairpin conformation $(CTG)_5$. Analysis of the transition kinetics reveals this occurs primarily through slowing of the opening transition.

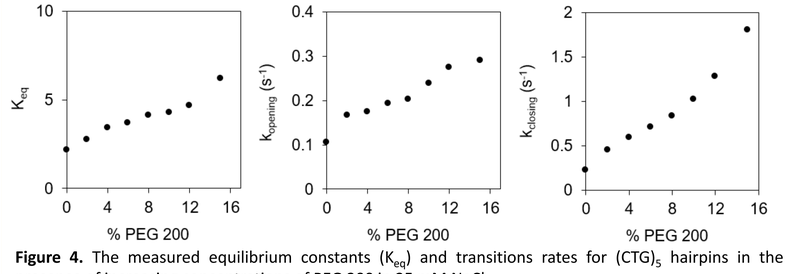

Figure 4. The measured equilibrium constants (K_{eq}) and transitions rates for $(CTG)_5$ hairpins in the presence of increasing concentrations of PEG 200 in 25 mM NaCl.

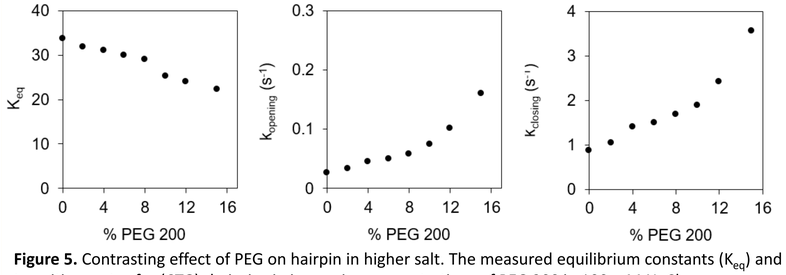

Figure 5. Contrasting effect of PEG on hairpin in higher salt. The measured equilibrium constants (K_{eq}) and transitions rates for $(CTG)_5$ hairpins in increasing concentrations of PEG 200 in 100 mM NaCl.

MOLECULAR CROWDING HAS ENTROPIC AND ENTHALPIC EFFECTS

To determine the thermodynamic parameters for the standard and transition states for the hairpins. We used a heated stage (Harvard Apparatus) to observe how the transitions kinetics of the hairpins changed over the temperature range 22 – 45 °C and quantify the enthalpic and entropic contributions to the formation and melting of TNR sequences with high self-complementarity and register degeneracy. The standard enthalpy and entropy values were determined according to van't Hoff analysis from the rate-derived equilibrium constants as

$$-Rln[K_{eq}] = \Delta H^o\left(\frac{1}{T}\right) - \Delta S^o$$

The transition state values for the hairpin to melted and the melted to hairpin pathways were calculated from the measured rates by the Eyring equation as

$$-Rln\left[\frac{k}{\nu}\right] = \Delta H^\dagger\left(\frac{1}{T}\right) - \Delta S^\dagger$$

where ν represents the collision frequency. Figure 5 shows the plots from this analysis for the $(CAG)_5$ hairpin. The extracted thermodynamic parameters are summarized for $(CAG)_5$ and $(CTG)_5$ in Table 1.

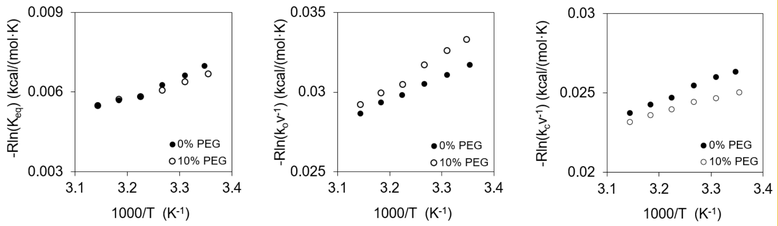

Figure 6. smFRET characterization of hairpin thermodynamics from transition kinetics for $(CTG)_5$ in 100 mM under different molecular crowding conditions. a) van't Hoff plot showing equilibrium versus inverse temperature. Eyring plots for the b) opening and c) closing transition rates versus inverse temperature. The thermodynamic parameters in Table 1 are derived from linear fits to the curves.

		Standard State			Opening Transition		Closing Transition	
Sequence	PEG 200	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol)/K	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol/K)
$(CTG)_5$	0%	-2.1	-8.9	-22.7	20.5	35.4	11.7	12.6
	10%	-2.0	-5.5	-12.0	14.4	16.5	8.8	4.5
$(CAG)_5$	0%	0.9	-9.4	-34.6	13.7	20.7	4.2	-13.9
	10%	1.1	0.6	-1.5	4.2	-8.7	4.9	-10.2

Table 1. Summary of standard and transition state thermodynamic parameters derived from temperature-based smFRET experiments for $(CTG)_5$ and $(CAG)_5$ hairpins at 100 mM NaCl in varying molecular crowding conditions.

CONCLUSIONS

- Increasing levels of monovalent salts promote stabilization of trinucleotide repeat hairpins. The stabilization occurs by slowing the opening transition. The slower acceleration of the closing rate for CTG with increasing NaCl mostly likely arises from misfolding.
- The misfolded species is supported by thermodynamic analysis of hairpin folding that reveals the formation of increased enthalpic and entropic barriers for the closing transition (1).
- Consistent with previous PEG-related studies on conformational transitions of RNA and DNA structures, increasing presence of PEG as a molecular crowding agent accelerates the closing transition through an entropic mediated pathway.
- For CTG and CAG repeat hairpins, molecular crowding accelerates the opening transition. This contrasts with previous reports that show molecular crowding to have little effect on the folded state. The findings support thermodynamic melting studies of trinucleotide repeat hairpins that demonstrate the presence of molecular crowding agent destabilizes the hairpins (Teng et al. 2018). The net effect on the equilibrium of folded species depends on the relative effects of PEG on the opening and closing transitions.
- The findings suggest that molecular crowding in the cell may confer some protective benefit by destabilizing formation of intrastrand structures with a high mismatch content.

REFERENCES

(1) Mitchell ML, Leveille MP, Solecki RS, Tran T, and Cannon B. Sequence-Dependent Effects of Monovalent Cations on the Structural Dynamics of Trinucleotide-Repeat DNA Hairpins. *Journal of Physical Chem. B.* 2018 Dec 20;122(50):11841-11851.